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(54) Title: METABOLIC ENGINEERING OF AMINO ACID PRODUCTION

(57) Abstract: The present invention is directed towards the fermentative production of amino acids, providing microorganisms, methods and processes useful therefor. Microorganisms of the invention are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with greater efficiency than the parent strain. The invention provides microorganisms that are made auxotrophic or bradytrophic for the synthesis of one or more branched chain amino acids by mutagenesis and selected for their ability to produce higher percent yields of the desired amino acid than the parental strain. Preferred microorganisms are Corynebacterium, Brevibacterium or Escherichia coli producing L-lysine. Mutagenesis is performed by classical techniques or through rDNA methodology. Methods of the invention are designed to increase the production of an amino acid by mutagenizing a parental strain, selecting cells auxotrophic or bradytrophic for the synthesis of one or more branched chain amino acids and selecting branched chain amino acid auxotrophs or bradytrophs that produce a higher percent yield from dextrose of the desired amino acid than the parental strain. Processes of the invention are designed for the production of an amino acid comprising culturing in a medium a microorganism obtained by mutagenizing a parent strain and selecting variants that are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with greater efficiency than the parent strain.

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Metabolic Engineering of Amino Acid Production

Background of the Invention

Field of the Invention

The invention relates to the areas of microbial genetics and recombinant DNA technology. More specifically, the present invention relates to the fermentative production of amino acids. The invention provides microorganisms useful for the production of amino acids, methods to increase the production of amino acids and processes for the production of amino acids.

Related Art

The production of amino acids through fermentation enables inexpensive production from cheap carbon sources such as molasses, acetic acid and ethanol. Following the recognition that *Corynebacteria* were useful for the industrial production of amino acids (S. Kinoshita *et al.*, *Proceedings of the International Symposium on Enzyme Chemistry* 2: 464-468 (1957)), commercial production of amino acids by fermentative processes was made more possible with the isolation of mutant strains. Microorganisms employed in microbial processes for amino acid production may be divided into 4 classes: wild-type strain, auxotrophic mutant, regulatory mutant and auxotrophic regulatory mutant (K. Nakayama *et al.*, in NUTRITIONAL IMPROVEMENT OF FOOD AND FEED PROTEINS, M. Friedman, ed., (1978), pp. 649-661). The stereospecificity of the amino acids produced by fermentation makes the process advantageous compared with synthetic processes; amino acids produced by microbial process are generally the L-form.

L-lysine is one example of an amino acid produced by industrial fermentation. Commercial production of this essential amino acid is principally done utilizing the gram positive *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum* (Kleemann, A., *et. al.*, Amino Acids,

in ULLMANN'S ENCYCLOPEDIA OF INDUSTRIAL CHEMISTRY, vol. A2, pp.57-97, Weinham: VCH-Verlagsgesellschaft (1985)); cumulatively, these three organisms presently account for the approximately 250,000 tons of L-lysine produced annually.

Given the economic importance of L-lysine production by fermentive processes, it would be beneficial to increase the total amount produced while simultaneously decreasing production costs. To that end, the biochemical pathway for L-lysine synthesis has been intensively investigated in *Corynebacterium* (recently reviewed by Sahm *et al.*, *Ann. N. Y. Acad. Sci. 782*: 25-39 (1996)). Entry into the lysine pathway begins with L-aspartate (see Figure 1), which itself is produced by transamination of oxaloacetate. A special feature of *C. glutamicum* is its ability to convert the lysine intermediate piperideine 2,6-dicarboxylate to diaminopimelate by two different routes, i.e. by reactions involving succinylated intermediates or by the single reaction of diaminopimelate dehydrogenase. Overall, carbon flux into the pathway is regulated at two points: first, through feedback inhibition of aspartate kinase by the levels of both L-threonine and L-lysine; and second through the control of the level of dihydrodipicolinate synthase. Therefore, increased production of L-lysine may be obtained in *Corynebacteria* by deregulating and increasing the activity of these two enzymes.

In addition to the biochemical pathway leading to L-lysine synthesis, recent evidence indicates that consideration of lysine transport out of cells into the media is another condition to be considered in the development of lysine over-producing strains of *C. glutamicum*. Studies by Krämer and colleagues indicate that passive transport out of the cell, as the result of a leaky membrane, is not the sole explanation for lysine efflux; their data suggest a specific carrier with the following properties: (1) the transporter possesses a rather high Km value for lysine (20mM); (2) the transporter is an OH^- symport system (uptake systems are H^+ antiport systems); and (3) the transporter is positively charged, and membrane potential stimulates secretion (S. Bröer and R. Krämer, *Eur. J. Biochem. 202*: 137-143 (1991).

Several fermentation processes utilizing various strains isolated for auxotrophic or resistance properties are known in the art for the production of L-lysine: U.S. Patent No. 2,979,439 discloses mutants requiring homoserine (or methionine and threonine); U.S. Patent No. 3,700,557 discloses mutants having a nutritional requirement for threonine, methionine, arginine, histidine, leucine, isoleucine, phenylalanine, cystine, or cysteine; U.S. Patent No. 3,707,441 discloses a mutant having a resistance to a lysine analog; U.S. Patent No. 3,687,810 discloses a mutant having both an ability to produce L-lysine and a resistance to bacitracin, penicillin G or polymyxin; U.S. Patent No. 3,708,395 discloses mutants having a nutritional requirement for homoserine, threonine, threonine and methionine, leucine, isoleucine or mixtures thereof and a resistance to lysine, threonine, isoleucine or analogs thereof; U.S. Patent No. 3,825,472 discloses a mutant having a resistance to a lysine analog; U.S. Patent No. 4,169,763 discloses mutant strains of *Corynebacterium* that produce L-lysine and are resistant to at least one of aspartic analogs and sulfa drugs; U.S. Patent No. 5,846,790 discloses a mutant strain able to produce L-glutamic acid and L-lysine in the absence of any biotin action-surpressing agent; and U.S. Patent No. 5,650,304 discloses a strain belonging to the genus *Corynebacterium* or *Brevibacterium* for the production of L-lysine that is resistant to 4-N-(D-alanyl)-2,4-diamino-2,4-dideoxy-L-arabinose 2,4-dideoxy-L-arabinose or a derivative thereof.

More recent developments in the area of L-lysine fermentive production in *Corynebacteria* involve the use of molecular biology techniques to augment lysine production. The following examples are provided as being exemplary of the art: U. S. Patent Application Nos. 4,560,654 and 5,236,831 disclose an L-lysine producing mutant strain obtained by transforming a host *Corynebacterium* or *Brevibacterium* microorganism which is sensitive to S-(2-aminoethyl)-cysteine with a recombinant DNA molecule wherein a DNA fragment conferring both resistance to S-(2-aminoethyl)-cysteine and lysine producing ability is inserted into a vector DNA; U. S. Patent Application No. 5,766,925 discloses a mutant strain produced by integrating a gene coding for aspartokinase, originating from coryneform bacteria, with desensitized feedback inhibition by L-lysine and

L-threonine, into chromosomal DNA of a *Coryneform* bacterium harboring leaky type homoserine dehydrogenase or a *Coryneform* bacterium deficient in homoserine dehydrogenase gene.

In addition to L-lysine, *Corynebacterium* and related organisms are useful for the production of other amino acids, for example the branched chain amino acids L-leucine, L-isoleucine and L-valine. The biochemical pathways leading to branched chain amino acid biosynthesis are also well studied. Carbon flux into the aspartate pathway may be funneled onto the production of L-lysine or L-threonine, which may be utilized for the production of L-isoleucine (Figure 1B). L-isoleucine is produced from L-threonine in five reactions; the enzymes catalyzing these reactions include: (1) threonine dehydratase; (2) acetohydoxy acid synthase; (3) isomeroreductase; (4) dihydroxy acid dehydratase; and (5) transaminase B. Threonine dehydratase is the only enzyme in this pathway unique to isoleucine synthesis; the other four enzymes are also utilized in the production of the other branched chain amino acids, valine and leucine. Carbon flux from threonine to isoleucine is controlled by threonine dehydratase and acetohydoxy acid synthase (AHAS). With the cloning of genes encoding the enzymes of the isoleucine pathway (*ilvA*, *ilvB*, *ilvC*, *ilvD* and *ilvE*) in *Corynebacterium* (C. Cordes *et al.*, *Gene* 112: 113-116 (1992); B. Möckel *et al.*, *J. Bacteriology* 174: 8065-8072 (1992); and C. Keilhauer *et al.*, *J. Bacteriology* 175: 5595-5603 (1993)), recombinant DNA techniques may be applied to generate novel strains.

Improvements in the production of the amino acids L-isoleucine, L-leucine and L-valine by increasing the activity of enzymes in the branched chain amino acid biosynthetic pathway have been described. Additionally, improvements in the production of branched chain amino acids by improving the acetohydroxy acid synthase (AHAS) activity encoded by the ilvBN operon have been described. (see generally H. Sahm *et al.*, *Ann. N. Y. Acad. Sci.* 782: 25-39 (1996)).

Exemplary processes for the production of branched chain amino acids include the following: U.S. Patent No. 5,188,948 discloses a fermentation process for producing L-valine utilizing a microorganism is resistant to a polyketide; U.S.

Patent No. 5,521,074 discloses a process for producing L-valine utilizing a microorganism which belongs to the genus *Corynebacterium* or *Brevibacterium*, which exhibits a) an ability to produce L-valine, b) resistance to L-valine in a medium containing acetic acid as a sole carbon source, and c) sensitivity to a pyruvic acid analog in a medium containing glucose as a sole carbon source; U.S. Patent No. 4,601,983 discloses a genetic sequence coding for the production of a protein having the activity of homoserine dehydrogenase capable of replication in coryneform bacteria and used to produce L-threonine and L-isoleucine; U.S. Patent No. 4,442,208 discloses a fermentation process for the production of L-isoleucine utilizing a *Brevibacterium* or *Corynebacterium* strain obtained by recombinant DNA techniques that is resistant to α-amino-β-hydroxy valeric acid; U.S. Patent No. 4,656,135 discloses a process for producing L-isoleucine, which comprises culturing a microorganism belonging to the genus *Brevibacterium* or the genus *Corynebacterium* which has a methyllysine resistance or α-ketomalonic acid resistance and which is capable of producing L-isoleucine in a liquid medium, and obtaining the accumulated L-isoleucine from said medium; U.S. Patent No. 5,118,619 discloses a method for the fermentative production of L-isoleucine from D,L-α-hydroxybutyrate by means of mutants that utilize D-lactate; U.S. Patent No. 5,763,231 discloses a process for producing L-leucine, which includes incubating a strain of the genus *Corynebacterium*, *Escherichia*, *Brevibacterium*, or *Microbacterium* in a culture medium and reacting the resulting cells with saccharides and acetic acid or its salt to form and accumulate L-leucine in the reaction solution; and U.S. Patent No. 3,970,519 discloses strains that resist feedback inhibition by leucine or its analogs and require at least one of isoleucine, threonine or methionine as a growth nutriment to produce L-leucine.

Improvements in the production of amino acids by decreasing the production of valine have not been described.

Improvements in the production of amino acids by decreasing AHAS activity have not been described.

Summary of the Invention

It is an object of the present invention to provide microorganisms that are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with greater efficiency than the parent strain. The efficiency of conversion may be quantified by the formula:

[(g amino acid produced/ g dextrose consumed)*100] = % Yield

and expressed as yield from dextrose.

In one embodiment, the invention provides microorganisms that are made auxotrophic for the synthesis of one or more branched chain amino acids by mutagenesis and selected for their ability to produce higher percent yields of the desired amino acid than the parental strain

In a more specific embodiment of the invention provides microorganisms obtained by subjecting a parental strain to random chemical mutagenesis, isolating a mutagenized variant that is auxotrophic for branched chain amino acid synthesis and selecting variants that are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with greater efficiency than the parent strain. Another specific embodiment of the invention provides microorganisms obtained by utilizing rDNA methodologies to introduce a change (*i.e.*, a mutation) in the nucleic acid sequence of the ilvBN operon, isolating a mutagenized variant that is auxotrophic or bradytrophic for branched chain amino acid synthesis and selecting variants that are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with greater efficiency than the parent strain.

In a preferred embodiment, the microorganisms of the invention produce L-lysine. Another preferred embodiment of the invention is drawn to *Corynebacterium* microorganisms, or *Brevibacterium* microorganisms, and particulary preferred microorganisms are *Corynebacterium* or *Brevibacterium* microorganisms that produce L-lysine. In a most preferred embodiment, the microorganisms have the identifying characteristics of NRRL No. B-30149 (also known as LC10) or NRRL No. B-30150 (also known as BF100-1030), strains

deposited on June 29, 1999 with the Agricultural Research Service Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 USA.

Another object of the invention provides methods to increase the production of an amino acid by mutagenizing a parental strain, selecting cells auxotrophic for the synthesis of one or more branched chain amino acids and selecting branched chain amino acid auxotrophs that produce a higher percent yield from dextrose of the desired amino acid than the parental strain.

In a preferred embodiment, the method is drawn to increasing the yield from dextrose of the amino acid L-lysine obtained by culturing *Corynebacterium* which, through random chemical mutagenesis or recombinant DNA (rDNA) technology, is made to be auxotrophic or bradytrophic for one or more of the branched chain amino acids leucine, isoleucine and valine.

In one specific embodiment, branched chain amino acid auxotrophy is the result of chemical mutangensis of *Corynebacterium*. In an alternative specific embodiment, branched chain amino acid auxotrophy is the result of mutatgenesis of the ilvBN operon by rDNA techniques.

Another object of the invention is to provide processes for the production of an amino acid from microorganisms that are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with greater efficiency than the parent strain.

In one embodiment, the invention provides a process for producing an amino acid comprising culturing in a medium a microorganism obtained by mutagenizing a parent strain to be auxotrophic or bradytrophic for branched chain amino acid synthesis and selecting variants that are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with greater efficiency than the parent strain.

In a preferred embodiment for the process, the microorganism utilized in fermentation is obtained by subjecting the parent strain to random chemical mutagenesis, isolating a mutagenized variant that is auxotrophic for branched chain amino acid synthesis and selecting variants that are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with

greater efficiency than the parent strain. In another preferred embodiment for the process, the microorganism utilized in fermentation is obtained by altering (*i.e.*, introducing a mutation) the nucleotide sequence of the ilvBN operon by rDNA methodology, isolating a mutagenized variant that is auxotrophic or bradytrophic for branched chain amino acid synthesis and selecting variants that are capable of converting glucose to amino acids other than L-isoleucine, L-leucine and L-valine with greater efficiency than the parent strain.

It is to be understood that the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed.

Brief Description of the Figures

Figure 1. (A) A schematic presentation of the biochemical pathway leading to L-lysine production in *Corynebacterium*; (B) A schematic presentation of the biochemical pathway leading to L-isoleucine production in *Corynebacterium*.

Figure 2. A-B) Presentation of the nucleotide sequence of the ilvBN operon of *Corynebacterium* (SEQ ID NO:1); C) Presentation of the amino acid sequence of the ilvBN operon of *Corynebacterium* (SEQ ID NO:2).

Figure 3. A-B) Presentation of the nucleotide sequence for the ilvBN deletion mutant in the plasmid pAL203Δ (SEQ ID NO:3); C) Presentation of the amino acid sequence for the ilvBN deletion mutant in the plasmid pAL203Δ (SEQ ID NO:4).

Figure 4. A-C) Presentation of the nucleotide sequence of the pRV1B5 allele (SEQ ID NO:5); D) Presentation of the amino acid sequence of the pRV1B5 allele (SEQ ID NO:6).

Detailed Description of the Preferred Embodiments

1. Definitions

In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Auxotroph: As used herein, the term auxotroph refers to a strain of microorganism requiring for growth an external source of a specific metabolite that cannot be synthesized because of an acquired genetic defect.

Amino Acid Supplement: As used herein, the term "Amino Acid Supplement" refers to an amino acid required for growth and added to minimal media to support auxotroph growth.

Bradytroph: As used herein, the term bradytroph refers to a strain of microorganism that exhibits retarded growth in the absence of an external source of a specific metabolite. A bradytroph can synthesize the metabolite, but because of an acquired genetic defect, the rate of synthesis is less than the parent strain's rate of synthesis of the same metabolite.

Branched Amino Acid: As used herein, the term refers to those amino acids in which the R group possesses a branched carbon structure, such as leucine, isoleucine and valine.

Carbon Flux: As used herein, the term refers to the movement of carbon between amphibolic, catabolic and/or anabolic biochemical pathways of an organism.

Chromosomal Integration: As used herein, the term refers to the insertion of an exogeneous DNA fragment into the chromosome of a host organism; more particularly, the term is used to refer to homologous recombination between an exogenous DNA fragment and the appropriate region of the host cell chromosome.

High Yield Derivative: As used herein, the term refers to strain of microorganism that produces a higher yield from dextrose of a specific amino acid when compared with the parental strain from which it is derived.

Mutation: As used herein, the term refers to a single base pair change, insertion or deletion in the nucleotide sequence of interest.

Operon: As used herein, the term refers to a unit of bacterial gene expression and regulation, including the structural genes and regulatory elements, in DNA. Examples of regulatory elements that are encompassed within the operon include, but are not limited to, promoters and operators.

Parental Strain: As used herein, the term refers to a strain of microorganism subjected to some form of mutagenesis to yield the microorganism of the invention.

Percent Yield From Dextrose: As used herein, the term refers to the yield of amino acid from dextrose defined by the formula [(g amino acid produced/ g dextrose consumed)*100] = % Yield.

Phenotype: As used herein, the term refers to observable physical characteristics dependent upon the genetic constitution of a microorganism.

Relative Growth: As used herein, the term refers to a measurement providing an assessment of growth by directly comparing growth of a parental strain with that of a progeny strain over a defined time period and with a defined medium.

Mutagenesis: As used herein, the term refers to a process whereby a mutation is generated in DNA. With "random" mutatgenesis, the exact site of mutation is not predictable, occurring anywhere in the genome of the microorganism, and the mutation is brought about as a result of physical damage caused by agents such as radiation or chemical treatment. rDNA mutagenesis is directed to a cloned DNA of interest, and it may be random or site-directed.

2. Microorganisms of the Invention Based On Decreased Carbon Flow To Branched Chain Amino Acid Synthesis And Increased Production of NonBranched Amino Acids

The invention provides generally for the creation of microorganisms that are auxotrophic for the branched chain amino acid synthesis in order to direct carbon flux to non-branched chain amino acid synthesis. More specifically, by selecting for a specific auxotrophic phenotype requiring one or more of the branched chain amino acids leucine, isoleucine or valine (*e.g.*, isoleucine and valine) or designing mutations in the ilvBN operon that decrease the flow of carbon to isoleucine, leucine and valine synthesis, carbon flux in the system may then become available for other metabolic pathways (*e.g.*, L-lysine synthesis).

In one specific embodiment, the invention provides a microorganism C that produces amino acid X, wherein said microorganism C is obtained by the following method:

(a) selecting a parental microorganism A that produces said amino acid from dextrose in percent yield Y;

(b) mutagenizing said parental microorganism A to produce microorganism B by a method selected from the group consisting of:

(i) random chemical mutagenesis; and

(ii) rDNA mutagenesis of the ilvBN operon;

(c) selecting from step (b) at least one mutagenized microorganism B that is auxotrophic or bradytrophic for one or more of the branched chain amino acids leucine, isoleucine and valine; and

(d) selecting from step (c) at least one microorganism C which produces said amino acid X from dextrose in percent yield Z, wherein said percent yield Z is greater than said percent yield Y.

The percent yield from dextrose is preferably calculated using the formula [(g amino acid/L / (g dextrose consumed/L)] *100.

Parental microorganisms may be selected from any microorganism known in the art that can produce amino acid X. Particularly favored parental microorganisms *Corynebacterium* and *Brevibacterium*.

The strains of the invention may be prepared by any of the methods and techniques known and available to those skilled in the art. Illustrative examples of suitable methods for constructing the inventive bacterial strains include but are not limited to the following: mutagenesis using suitable agents such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG); gene integration techniques, mediated by transforming linear DNA fragments and homologous recombination; and transduction mediated by a bacteriophage. These methods are wéll known in the art and are described, for example, in J.H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972); J.H. Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1992); M. Singer and P. Berg, *Genes & Genomes*, University Science Books, Mill Valley, California (1991); J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); P.B. Kaufman *et al.*, *Handbook of Molecular and Cellular Methods in Biology and Medicine*, CRC Press, Boca Raton, Florida (1995); *Methods in Plant Molecular Biology and Biotechnology*, B.R. Glick and J.E. Thompson, eds., CRC Press, Boca Raton, Florida (1993); and P.F. Smith-Keary, *Molecular Genetics of Escherichia coli*, The Guilford Press, New York, NY (1989).

A. Construction of Branched Chain Amino Acid Auxotrophs by Random Mutagenesis

One specific preferred embodiment of the invention provides that modification of an enzymatic step common to L-isoleucine, L-leucine and L-valine biosynthesis can increase the percent yield of L-lysine from dextrose.

In a most preferred embodiment, the invention provides for the production of microorganisms that are auxotrophic for branched chain amino acid synthesis by means of random mutagenesis of a parental strain followed by selection of the

specific phenotype. The parental strain chosen for mutagenesis may be any strain known to produce the amino acid of interest. Preferred organisms include *Corynebacterium* strains and *Brevibacterium* strains, and most preferred organisms include *Corynebacterium* strains and *Brevibacterium* strains that produce L-lysine.

The parental strain may be mutagenized using any random mutagenesis technique known in the art, including, but not limited to, radiation and chemical procedures. Particularly preferred is random chemical mutagenesis, and most preferable is the alkylating agent method described by J. H. Miller (J. H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory (1972).

By way of example, chemical mutagenesis was conducted as follows. A culture of lysine-producing Corynebacterium strain was grown in rich medium at 30°C up to an optical density of 6.0. Cells were washed with minimal medium and resuspended in minimal medium containing 100 micrograms per mL of NTG. Cells were exposed to the mutagen for 30 minutes at 30°C. Cells were washed with minimal medium and plated onto rich medium. Colonies from rich medium were replica-plated to rich and minimal medium. Colonies that grew on rich medium but did not grow on minimal medium were classified as auxotrophs. Auxotroph mutants were replica-plated onto minimal medium and minimal medium containing 10 mM L-isoleucine and 10 mM L-valine. Colonies that were rescued by the isoleucine and valine were classified as valine auxotrophs. Strain B4B is a valine auxotroph generated by chemical mutagenesis.

B. Construction of Branched Chain Amino Acid Auxotrophs by Mutagenesis Through rDNA Methodology

Another specific preferred embodiment of the invention utilizes recombinant DNA technology to effect *in vitro* and *in vivo* mutagenesis of cloned DNA sequences that encode proteins important for the biosynthesis of branched chain amino acids. The mutated DNA may then be used to modify the parental strain to produce mutant strains that are auxotrophic for branched chain amino

acid synthesis and that produce a higher yield from dextrose of non-branched chain amino acids than the parental strain.

In one specific preferred embodiment, the cloned DNA of interest may be mutated through recombinant DNA technology by any means known in the that art. As one skilled in the art would know, the mutations in the cloned DNA may constitute single nucleotide changes (point mutations), multiple nucleotide changes, nucleotide deletions or insertions. General methods for recombinant DNA technology are known to those skilled in the art and may be found in a number of common laboratory manuals that describe fundamental techniques, such as nucleic acid purification, restriction enzyme digestion, ligation, gene cloning, gene sequencing, polymerase chain amplification (PCR) of gene sequences, and the like. (*see e.g.*, Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989); Current Protocols in Molecular Biology. Ausubel *et al.* eds., John Wiley & Sons, New York, (1994); *PCR Protocols*, Innis *et al.*, eds., Academic Press, Inc., New York, pp. 407-415 (1990)).

In addition, references that specifically teach *in vitro* mutatgenesis of cloned DNA are known to those skilled in the art. For example, strategies such as site-directed mutatgenesis, oligonucleotide-directed mutatgenesis, linker scanning mutatgenesis, random chemical mutatgenesis *in vitro*, cassette mutatgenesis, PCR mutatgenesis and others are detailed in *Directed Mutagenesis: A Practical Approach*, M. J. McPherson, ed., Oxford University Press, New York, (1991).

In another specific preferred embodiment, the cloned DNA of interest may be mutated *in vivo* in a host cell. This type of "*in vivo* mutagenesis" includes processes of generating random mutations in any cloned DNA of interest by the propagation of the DNA in a strain of *E. coli* that carries mutations in one or more of the DNA repair pathways. These "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will generate random mutations within the DNA. Systems designed to accomplish this are known to those skilled in the art and are available

commercially. For example, Stratagene, Inc. provides a system utilizing the XL1 Red Strain of *E. coli* which has had its DNA repair genes (MutH, MutL and MutS) deleted such that many different mutations occur in a short time. Up to 10,000 mutations may take place within a 30 hour time span such that an entire mutated DNA library may be prepared from mutated DNA by procedures known in the art.

The cloned DNA selected for mutation may be any sequence known in the art to be important for branched chain amino acid synthesis, including but not limited to, sequences encoding one or more enzymes important for synthesis or one or more protein products important for transport and excretion. Most preferred is the cloned sequence for the ilvBN operon of *Corynebacterium* or *Brevibacterium. Corynebacterium* genes involved in branched chain amino acid synthesis have been cloned; for example, gene sequences are available for the isoleucine pathway (ilvA, ilvB, ilvC, ilvD and ilvE) (C. Cordes *et al.*, *Gene* 112: 113-116 (1992); B. Möckel *et al.*, *J. Bacteriology* 174: 8065-8072 (1992); and C. Keilhauer *et al.*, *J. Bacteriology* 175: 5595-5603 (1993)).

L-isoleucine is produced from L-threonine in five reactions; the enzymes catalyzing these reactions include: (1) threonine dehydratase (ilvA); (2) acetohydoxy acid synthase (ilvBN); (3) isomeroreductase (ilvC); (4) dihydroxy acid dehydratase (ilvD); and (5) transaminase B (ilvE). Threonine dehydratase is the only enzyme in this pathway unique to isoleucine synthesis; the other four enzymes are also utilized in the production of the other branched chain amino acids, valine and leucine. The enzymatic pathway involved in isoleucine biosynthesis in *Corynebacterium* strains is presented in Figure 2.

Acetohydroxy acid synthase (AHAS) and isomeroreductase (IR) catalyze subsequent reactions in the flux of metabolites towards isoleucine, valine, leucine, and pantothenate. As in other bacteria, the AHAS of *Corynecacterium* strains is encoded by two genes, ilvB and ilvN. Gene disruption verified that these genes encode the single AHAS activity in *C. glutamicum* (Keilhauer, C., et al., *J. Bacteriology* 175:5595-5603 (1973)). Three transcripts of 3.9, 2.3, and 1.1 kb were identified *in vivo* by Northern Blot analysis, which correspond to ilvBNC,

ilvNC, and ilvC messages, respectively. The ilvC transcript (encoding IR) is the most abundant transcript from the ilv operon of *C. glutamicum*. Additional analysis indicates that three promoters are active in this operon; the steady-state levels of the ilvBNC and ilvNC messages contribute significantly to the total activity of the single AHAS.

In a most preferred invention embodiment, a mutation may be generated by way of restriction enzyme digestion to create a deletion in the cloned ilvBN operon DNA sequence. The mutated ilvBN sequence may then be sustituted for the wild type sequence by homologous recombination and screened for branched chain amino acid auxotrophy.

Another embodiment of the invention is drawn to a microorganism *Corynebacterium* having the following that is auxotrophic for the one or more of the branched chain amino acids isoleucine, leucine and valine and produces a percent yield from dextrose of the an amino acid of interest that is greater than the parental strain percent yield. In a particularly favored embodiment, the amino acid produced is L-lysine.

Other highly preferred embodiments of the invention are drawn to microorganisms having substantially all of the characteristics of NRRL Deposit No. B-30149 or NRRL Deposit No. B-30150.

3. Methods of Increasing the Production of an Amino Acid

A further object of the invention provides methods to increase the production of an amino acid. The invention provides generally for a method to increase the production of an amino acid X, comprising:

(a) selecting a parental microorganism A that produces said amino acid from dextrose in percent yield Y;

(b) mutagenizing said parental microorganism A to produce microorganism B by a method selected from the group consisting of:

(i) random chemical mutagenesis; and

(ii) rDNA mutagenesis of the ilvBN operon;

(c) selecting from step (b) at least one mutagenized microorganism B that is auxotrophic for one or more of the branched chain amino acids leucine, isoleucine and valine; and

(d) selecting from step (c) at least one microorganism C which produces said amino acid X from dextrose in percent yield Z, wherein said percent yield Z is greater than said percent yield Y.

In one particular preferred embodiment, any strain known in the art may be selected as a parental strain that produces the amino acid of interest at a determined percent yield from dextrose. The percent yield from dextrose may be easily calculated using the following formula: [(g amino acid/L / (g dextrose consumed/L)] *100.

After selecting the organism and determining the percent yield from dextrose of the amino acid, the microorganism is preferably subjected to mutagenesis either by random mutagenesis techniques directed at the entire genome of the organism or by rDNA techniques directed towards cloned DNA of interest. Regardless of the particular method of mutagenesis employed, mutated organisms are screened and selected on the basis of auxotrophy for branched chain amino acid synthesis. Auxotrophs selected may then be screened to determine which strains produce a higher percent yield of the desired amino acid from dextrose than the parental strain.

Various embodiments of the invention include methods to increase the production of an amino acid of interest from the organisms *Corynebacterium*, *Brevibacterium*, and *E. coli*. Additionally, depending upon the particular embodiment, the invention provides methods to increase the production of non-branched amino acids, such as glycine; alanine; methionine; phenylalanine; tryptophan; proline; serine; threonine; cysteine; tyrosine; asparagine; glutamine; aspartic acid; glutamic acid; lysine; arginine; and histidine.

In a favored embodiment, the invention provides methods to increase non-branched chain amino acid production by creating auxotrophs for branched chain amino acid synthesis and diverting carbon flux from the synthesis of leucine,

isoleucine and valine. A particularly favored embodiment is drawn to a method of increasing the production of an amino acid by selecting from a mutagenized parental strain a strain that is auxotrophic for valine and isoleucine synthesis.

The invention further provides various preferred embodiments for methods to increase the production of an amino acid wherein the parental strain may be mutagenized either by random mutagenesis techniques (*e.g.*, radiation or chemical mutagenesis) or mutagenesis of the ilvBN operon by rDNA techniques. In one particular preferred embodiment, the parental strain may be mutagenized by random chemical mutagenesis. In another particular preferred embodiment, the parental strain is mutagenized by rDNA techniques directed at the cloned ilvBN operon nucleotide sequence.

4. Processes for the Production of an Amino Acid

A further object of the invention provides processes for the production of an amino acid. The invention provides generally for a process for producing an amino acid X comprising:

(a) culturing a microorganism C in a medium, wherein said microorganism C is obtained by the following method:

(i) selecting a parental microorganism A that produces said amino acid from dextrose in percent yield Y;

(ii) mutagenizing said parental microorganism A to produce microorganism B by a method selected from the group consisting of:

(1) random chemical mutagenesis; and

(2) rDNA mutagenesis of the ilvBN operon;

(iii) selecting from step (b) at leat one mutagenized microorganism B that is auxotrophic for one or more of the branched chain amino acids leucine, isoleucine and valine; and

(iv) selecting from step (c) at least one microorganism C which produces said amino acid X from dextrose in percent yield Z, wherein said percent yield Z is greater than said percent yield Y; and

(b) recovering said amino acid X that is produced from said microorganism C.

Preferred embodiments of the invention are drawn to processes in which the cultured microorganism is selected from the group that includes *Corynebacterium*, *Brevibacterium*, and *E. coli*. Particularly preferred are process drawn to the organisms of the genus *Corynebacterium*. Microorganisms selected for the processes of the invention are those that produce an amino acid of interest, particularly non-branched chain amino acids. More particularly preferred microorganisms are microorganisms that produce glycine; alanine; methionine; phenylalanine; tryptophan; proline; serine; threonine; cysteine; tyrosine; asparagine; glutamine; aspartic acid; glutamic acid; lysine; arginine; and histidine. The level of production of the amino acid of choice may conveniently determined by the following formula to calculate the percent yield from dextrose: [(g amino acid/L / (g dextrose consumed/L)] *100.

Microorganisms used in the processes of the invention are preferably obtained by mutatgenesis of the chosen parental strain. Preferred embodiments of the invention include processes in which the chosen parental strains are subjected either to random mutagenesis directed at the entire genome or to rDNA mutagenesis of cloned DNA of interest.

Particularly preferred embodiments of the invention wherein the parental strain is subjected to random mutagenesis include but are not limited to, mutagenesis by radiation treatment or chemical treatment. A more particularly preferred embodiment is drawn to random chemical mutagenesis of the parental strain.

Another particularly preferred embodiment of the invention provides for rDNA mutagenesis of the parental strain. A more particularly preferred embodiment is drawn to rDNA mutagenesis of the ilvBN operon *in vitro* or *in*

vivo. Mutated forms of the ilvBN operon, or fragments thereof, may then be substituted for wild-type ilvBN operon sequence through homologous recombination techniques that are well known to those skilled in the art (see Example 6).

However the selected parental strains or cloned DNA sequences are mutagenized, the resultant progeny are screened and selected for auxotrophy for branched chain amino acid synthesis (*i.e.*, leucine, isoleucine or valine). The selection of such mutants is well with in the skill of those in the art. A particularly preferred embodiment is drawn to strains that are auxotrophic for valine and isoleucine biosynthesis.

Ultimately, selection of the microorganisms of the processes of the invention is dependent upon production of the amino acid of choice. Utilizing the formula [(g amino acid/L / (g dextrose consumed/L)] *100 to determine the percent yield from dextrose, the desired microorganisms are selected on the basis of having a higher percent yield from dextrose of the amino acid of choice than the parental strain.

Other embodiments of the invention are drawn to processes that vary by way of the specific method of culturing the microorganisms of the invention. Thus, a variety of fermentation techniques are known in the art which may be employed in processes of the invention drawn to the production of amino acids.

Illustrative examples of suitable carbon sources include, but are not limited to: carbohydrates, such as glucose, fructose, sucrose, starch hydrolysate, cellulose hydrolysate and molasses; organic acids, such as acetic acid, propionic acid, formic acid, malic acid, citric acid, and fumaric acid; and alcohols, such as glycerol.

Illustrative examples of suitable nitrogen sources include, but are not limited to: ammonia, including ammonia gas and aqueous ammonia; ammonium salts of inorganic or organic acids, such as ammonium chloride, ammonium phosphate, ammonium sulfate and ammonium acetate; and other nitrogen-containing, including meat extract, peptone, corn steep liquor, casein hydrolysate, soybean cake hydrolysate and yeast extract.

Generally, amino acids may be commercially produced from the invention in fermentation processes such as the batch type or of the fed-batch type. In batch type fermentations, all nutrients are added at the beginning of the fermentation. In fed-batch or extended fed-batch type fermentations one or a number of nutrients are continuously supplied to the culture, right from the beginning of the fermentation or after the culture has reached a certain age, or when the nutrient(s) which are fed were exhausted from the culture fluid. A variant of the extended batch of fed-batch type fermentation is the repeated fed-batch or fill-and-draw fermentation, where part of the contents of the fermenter is removed at some time, for instance when the fermenter is full, while feeding of a nutrient is continued. In this way a fermentation can be extended for a longer time.

Another type of fermentation, the continuous fermentation or chemostat culture, uses continuous feeding of a complete medium, while culture fluid is continuously or semi-continuously withdrawn in such a way that the volume of the broth in the fermenter remains approximately constant. A continuous fermentation can in principle be maintained for an infinite time.

In a batch fermentation an organism grows until one of the essential nutrients in the medium becomes exhausted, or until fermentation conditions become unfavorable (e.g. the pH decreases to a value inhibitory for microbial growth). In fed-batch fermentations measures are normally taken to maintain favorable growth conditions, e.g. by using pH control, and exhaustion of one or more essential nutrients is prevented by feeding these nutrient(s) to the culture. The microorganism will continue to grow, at a growth rate dictated by the rate of nutrient feed. Generally a single nutrient, very often the carbon source, will become limiting for growth. The same principle applies for a continuous fermentation, usually one nutrient in the medium feed is limiting, all other nutrients are in excess. The limiting nutrient will be present in the culture fluid at a very low concentration, often unmeasurably low. Different types of nutrient limitation can be employed. Carbon source limitation is most often used. Other examples are limitation by the nitrogen source, limitation by oxygen, limitation by a specific nutrient such as a vitamin or an amino acid (in case the microorganism is

auxotrophic for such a compound), limitation by sulphur and limitation by phosphorous.

The amino acid may be recovered by any method known in the art. Exemplary procedures are provided in the following: Van Walsem, H.J. & Thompson, M.C., *J. Biotechnol. 59*:127-132 (1997), and U.S. Patent No. 3,565,951, both of which are incorporated herein by reference.

All patents and publications referred to herein are expressly incorporated by reference.

Examples

Example 1

Chemical Mutagenesis and Selection of Valine Auxotrophs

A lysine producing *Corynebacterium* strain BF100 was mutagenized with an alkylating agent as described in Miller, J.H. 1972 (Miller, J.H. 1972 Experiments in Molecular Genetics. Cold Spring Harbor Laboratory). Colonies were replica plated onto minimal medium (MM). Those that did not grow on MM but grew on complete medium (CM) were identified as auxotrophs. Those auxotrophs that were capable of growth on MM when supplemented with L-valine and L-isoleucine were selected for lysine yield analysis.

MM consisted of 20 g D-glucose, 10 g ammonium sulfate, 2.5 g urea, 1 g KH2PO4, 0.4 g MgSO4.7H20, 1 g NaCl, 0.01 g MnSO4.H20, 0.01 g FeSO4.7H20, 10 mg pantothenate, 50 mg biotin, 200 mg thiamine, and 50 mg niacinamide per liter at pH 7.2. When L-amino acids were used to supplement MM, 50 mg/L of each was used. MMIV was MM with isoleucine and valine added.

The growth pattern of a parent strain and a high yield-derivative produced by chemical mutagenesis on minimal agar plates supplemented with three amino acids is presented in Table 1. Supplements are at 50mg/L L-amino acids. Growth is presented as relative colony size after 3 days at 30C.

Example 2

Production of Branched Chain Auxotrophs with rDNA Technology

1. Preparation of a Deleted ilvB Gene

The ilvBN operon of *Corynebacterium lactofermentum* (ATCC 21799) was amplified by PCR and cloned into pCR-Script to make pAL203. The ilvB gene contains a 390 bp region separated by 2 EcoNI restriction sites. EcoNI does not cut the plasmid pCR-Script. The ilvB deletion allele was designed by cutting the plasmid pAL203 with EcoNI followed by selfligation to yield pAL203delta.

2. Homologous Recombination of a Modified ilvBN Allele Into the Corynebacterium Chromosome

A vector for allele exchange by double crossover was constructed as described by Maloy et al. 1996 (Maloy S.R. , Stewart V. J., and Taylor R. K. 1996 Genetic Analysis of Pathogenic Bacteria: A Laboratory Manual, Cold Spring Harbor Press). ATCC 37766 was the source of pK184 a plasmid that replicates in *E. coli* but not in *Corynebacterium*. A sacB gene was subcloned into its SspI site to give pJC3. pJC3 cannot replicate in *Corynebacterium*. Any kanamycin resistant colonies will have the vector integrated into the chromosome by homologous recombination at a site within the cloned gene. Lethal expression of the sacB gene on the integrated vector prevents growth in the presence of sucrose. Growth in the presence of sucrose requires a second cross over to occur along an homologous region of the cloned insert. If the first and second crossovers flank a modification (deletion, site mutation) , then the modified allele of ilvBN will be exchanged for the allele present on the chromosome of the host strain.

The modified allele of the ilvBN operon from pAL203Δ was subcloned into the integration vector pJC3 and electroporated into the BF100 strain of *Corynebacterium* and plated on rich medium plates lacking sucrose but having kanamycin (DMK). Colonies were picked and grown in rich broth lacking sucrose and kanamycin for 48 hrs. Cultures were streaked onto rich plates lacking

kanamycin but having sucrose. Single colonies were picked from sucrose plates and replica plated on DMK, SM1, MM and MMIV. Strains that had no kanamycin resistance, could grow on sucrose, and could not grow on MM but could grow on MMIV were selected for shake flask experiments. LC10 is a BF100-derived auxotroph.

The growth pattern of a parent strain and a high yield-derivative produced with recombinant DNA methods on a series of minimal agar plates supplemented with three amino acids is presented in Table 2. Supplements are at 50mg/L L-amino acids. Growth is presented as relative colony size after 3 days at 30C.

Example 3

Shake Flask Determination of L-lysine Yield From Valine Auxotroph Strain Produced by Random Chemical Mutagenesis

B4B inoculum was prepared by picking a single colony from an SM1 plate and transferring to SM1 broth. SM1 was made by combining 50g sucrose, 3 g K2HP04, 3 g urea, 0.5 g MgSO4.7H20, 20 g polypeptone, 5 g beef extract, 0.9 mg D-biotin, 3 mg thiamine, 125 mg niacinamide, 0.5 g L-methionine, 0.25 g L-threonine, 0.5 g Lalanine per liter of water and adjusting the pH to 7.3. Plates included 20 g/L agar. After 16 hr growth of cultures in SM1 broth, an equal volume of 30% glycerol was added and cultures were frozen at -80C.

Baffled 250 mL seed shake flasks with 20 mL of SFM were inoculated with 0.1 mL of thawed inoculum. Seed medium (SFM) consisted of 60 g D-glucose, 3 g K2HP04, 3 g urea, 0.5 g MgSO4.7H20, 20 g polypeptone, 5 g beef extract, 3 mg D-biotin, 125 mg niacinamide, 0.5 g L-methionine, 0.25 g L threonine, and 0.5 g L-alanine per liter of water with pH adjusted to 7.3. Cultures were grown at 30C for 16 hrs and aerated at 240 rpm with a 2 inch displacement. Two mL of seed culture was used to inoculate 21 mL of fermentation medium (FM4). FM4 medium was made by mixing 16 mL of main medium with 5 mL of dextrose stock. Dextrose stock was 180 g D-glucose plus 500 mL water. Main medium contained 0.083 g MnSO4, 0.4 mg D-biotin, cornsteep liquor, raffinate and 50 g CaCO3 per liter. Cornsteep was added so

that the final volume of FM4 was 4% dry solids. Raffinate was added so that the final volume of FM4 had 5% ammonium sulfate. Cultures were grown for 48 hrs at 30C in 250 mL baffled shake flasks and aerated at 240 rpms with a 2 inch displacement.

Table 3 presents data on the production of L-lysine in shake flasks by *Corynebacterium* strain improved by selection for valine and isoleucine requirement.

Example 4

Shake Flask Determination of L-lysine Yield From Valine Auxotroph Strain Produced by rDNA Methodology

Table 4 presents data on the production of L-lysine in shake flasks by *Corynebacterium* strains improved by deleting 390 bases of DNA sequence from the chromosomal copy of the i1vBN operon (see Example 2). Cultures were grown and analyzed as described in Example 3.

Example 5

Microfermenation Determination of Lysine Yield by Valine Auxotroph

Inoculum was grown in 500 mL SM1 in a 2 L baffled shake flask for 18 hrs. 3.1 L of FM4 medium was used in 4 L microfermentors. Temperature and pH were maintained at 32C and 7.2, respectively. Agitation was increased from 700 rpms to 950 rpms at 20 hrs. Air was fed at 4.5 LPM. Dextrose was maintained at 3 g/L. Fermentation time was 48 hrs.

Table 5 presents data on the production of L-lysine in 4 liter fermentors using a strains of *Corynebacterium* which cannot synthesize L-isoleucine and L-valine.

Example 6

L-Lysine Production by Bradytroph Produced by In Vivo Mutagenesis of Cloned ILVBN

1. Preparation of a Defective ilvBN Operon that Produces a Functional AHAS Enzyme

The ilvBN operon of pAL203 was subcloned into the shuttle vector pM2 to give pVAL1. pM2 can replicate in both *E. coli* and *Corynebacterium.* pVal1 was transformed into the mutagenic strain XL1RED from the Stratagene Co. Mutagenized plasmid was prepared according to the XL1RED kit instructions and electroporated into a valine auxotroph, *Corynebacterium.* A valine auxotroph is unable to grow on MM plates without supplementation by isoleucine and valine or genetic complementation with a functional ilvBN operon.

Kanamycin resistant transformants were selected from SM1 plates and replica plated on to MM plates. Those colonies that grew on MM plates showed functional complementation of the ilvB deletion. Colonies that were smaller than the colonies of the valine auxotroph with the parent plasmid (pVAL1) were selected for the valine auxotroph activity assays. pRV1B5 is a plasmid derived from pVAL1 that can replicate in *E. coli* and *Corynebacterium.* In the valine auxotroph strain, it produced AHAS activity at less than 1% of the specific activity of AHAS produced by pVAL1. The ilvBN operon of this construct has leaky AHAS activity.

2. Homologous Recombinantion into Corynebacterium Chromosomal DNA

The RV1B5 leaky allele of the ilvBN operon was subcloned into the integration vector pJC3 and used to exchange the leaky allele for the deletion allele in a valine auxotroph by homologous recombination as done in Example 2. BF100-1030 is a valine bradytroph constructed with the RV1B5 allele. Table 6 presents data showing that BF100-1030 makes less valine in shake flasks than its parent strain. Table 7 shows that BF100-1030 bradytroph has improved growth

over the auxotrophs in Table 5. Table 7 also shows that the bradytroph produces less valine in microfermentors than the parent strain.

Tables

Data presented in the following tables are discussed in the Examples section. Note that the term "Growth" refers to the optical density measured at 660 nm; the term "Titre" refers to the grams of amino acid per liter; the term "Yield" is defined by the following formula: [(g lysine/L / (g dextrose consumed/L)] *100; B4B = a valine autotroph constructed with chemical mutagenesis; LC10 = a valine autotroph constructed by replacing the chromosomal ilvB gene with the ilvB deletion allele of pAL203Δ; BF100-1030 = a valine bradytroph constructed by replacing the chromosomal ilvB gene with the RV1B5 leaky allele.

Table 1
Valine Auxotroph Selection Following Chemical Mutagenesis

	Amino Acid Supplement			Relative Growth	
Agar Plate	ile	leu	val	BF100	B4B
MM	-	-	-	5	0
MM	+	+	-	5	0
MM	+	-	+	5	2
MM	-	+	+	5	0
MM	+	+	+	5	5

Table 2
Auxotroph Selection Following rDNA Modification

	Amino Acid Supplement			Relative Growth	
Agar Plate	ile	leu	val	BF100	LC10
MM	-	-	-	3	0
MM	+	+	-	3	0
MM	+	-	+	3	1
MM	-	+	+	3	0
MM	+	+	+	3	3

Table 3
Shake Flask Determination of L-lysine Yield From a Valine Auxotroph Strain Produced by Random Chemical Mutagenesis

Strain	Growth	Lysine Titre	% Yield
Parent-1	33	18	32
B4B	33	17	44

Table 4
Shake Flask Determination of L-lysine Yield From a Valine Auxotroph Strain Produced by rDNA Methodology

Strain	Growth	Lysine Titre	% Yield
BF100	35	25	38
LC10	35	28	43

Table 5
Microfermenation Determination of Lysine Yield

Strain	Growth	Lysine Titre	% Yield	Valine Titre
BF100	90	113	37	8.9
LC10	63	86	50	1.1
B4B	70	91	51	---

Table 6

Shake Flask Determination of Decreased L-Valine Titre from a Valine Bradytroph Strain Produced by Integrating the RV1B5 allele of ilvB into the Chromosome

Strain	Growth	Lysine Titre	% Yield	Valine Titre
BF100	36	27	29	5.2
BF100-1030	42	22	25	3.3

Table 7

Microfermentation Determination of Decreased L-Valine Titre from a Valine Bradytroph Strain Produced by Integrating the RV1B5 Allele of ilvB into the Chromosome

Strain	Growth	Lysine Titre	% Yield	Valine Titre
BF100	89	134	43	9
BF100-1030	78	123	44	6

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 5, line 28.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depository institution

Agricultural Research Culture Collection (NRRL)

Address of depository institution *(including postal code and country)*

1815 N. University Street
Peoria, Illinois 61604
United States of America

Date of deposit	Accession Number
June 29, 1999	NRRL B-30149

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Corynebacterium glutamicum LC10

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
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A. The indications made below relate to the microorganism referred to in the description on page 5, line 29.

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1815 N. University Street
Peoria, Illinois 61604
United States of America

Date of deposit	Accession Number
June 29, 1999	NRRL B-30150

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Corynebacterium glutamicum BF100-1030

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

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What Is Claimed Is:

1. A microorganism C that produces amino acid X, wherein said microorganism C is obtained by the following method:

(a) selecting a parental microorganism A that produces said amino acid from dextrose in percent yield Y;

(b) mutagenizing said parental microorganism A to produce microorganism B by a method selected from the group consisting of:

(i) random chemical mutagenesis; and

(ii) rDNA mutagenesis of the ilvBN operon;

(c) selecting from step (b) at least one mutagenized microorganism B that is auxotrophic or bradytrophic for one or more of the branched chain amino acids leucine, isoleucine and valine; and

(d) selecting from step (c) at least one microorganism C which produces said amino acid X from dextrose in percent yield Z, wherein said percent yield Z is greater than said percent yield Y.

2. The microorganism C of Claim 1, wherein amino acid X is selected from the group consisting of:

(a) glycine;

(b) alanine;

(c) methionine;

(d) phenylalanine;

(e) tryptophan;

(f) proline;

(g) serine;

(h) threonine;

(i) cysteine;

(j) tyrosine;

(k) asparagine;

(l) glutamine;

(m) aspartic acid;

(n) glutamic acid;

(o) lysine;

(p) arginine; and

(q) histidine.

3. The microorganism C of Claim 2, wherein microorganism A is selected from the group consisting of:

(a) *Corynebacterium*;

(b) *Brevibacterium*; and

(c) *E. coli*.

4. The microorganism C of Claim 3, wherein microorganism B is auxotrophic or bradytrophic for valine and isoleucine.

5. The microorganism C of Claim 3, wherein step (ii) mutagenesis is random chemical mutagenesis.

6. The microorganism C of Claim 3, wherein step (ii) mutagenesis is site-specific mutagenesis of the ilvBN operon.

7. A strain of the microorganism *Corynebacterium* having the following characteristics:

(a) auxotrophy or bradytrophy for the one or more of the branched chain amino acids isoleucine, leucine and valine; and

(b) when cultured in a medium, produces an amino acid selected from the group consisting of:

(i) glycine;

(ii) alanine;

(iii) methionine;

(iv) phenylalanine;

(v) tryptophan;
(vi) proline;
(vii) serine;
(viii) threonine;
(ix) cysteine;
(x) tyrosine;
(xi) asparagine;
(xii) glutamine;
(xiii) aspartic acid;
(xiv) glutamic acid;
(xv) lysine;
(xvi) arginine; and
(xvii) histidine;

and

(c) produces a percent yield from dextrose of the amino acid of step (b) of at least about 30 percent.

8. The microorganism of Claim 7, further characterized by a mutation in the ilvBN operon.

9. The microorganism of Claim 8, wherein the ilvBN operon mutation is a deletion, insertion or point mutation.

10. The microorganism of Claim 9, wherein the ilvBN operon mutation is characterized by the sequence of SEQ ID NO:3 or SEQ ID NO:5.

11. A strain of the microorganism *Corynebacterium* having the identifying characteristics of NRRL Deposit No. B-30149.

12. A strain of the microorganism *Corynebacterium* having the identifying characteristics of NRRL Deposit No. B-30150.

13. A method to increase the production of an amino acid X, comprising:

(a) selecting a parental microorganism A that produces said amino acid from dextrose in percent yield Y;

(b) mutagenizing said parental microorganism A to produce microorganism B by a method selected from the group consisting of:

(i) random chemical mutagenesis; and

(ii) rDNA mutagenesis of the ilvBN operon;

(c) selecting from step (b) at least one mutagenized microorganism B that is auxotrophic or bradytrophic for one or more of the branched chain amino acids leucine, isoleucine and valine; and

(d) selecting from step (c) at least one microorganism C which produces said amino acid X from dextrose in percent yield Z, wherein said percent yield Z is greater than said percent yield Y.

14. The method of Claim 13, wherein amino acid X is selected from the group consisting of:

(a) glycine;

(b) alanine;

(c) methionine;

(d) phenylalanine;

(e) tryptophan;

(f) proline;

(g) serine;

(h) threonine;

(i) cysteine;

(j) tyrosine;

(k) asparagine;

(l) glutamine;

(m) aspartic acid;

(n) glutamic acid;

(o) lysine;

(p) arginine; and

(q) histidine.

15. The method of claim 14, wherein microorganism A is selected from the group consisting of:

(a) *Corynebacterium*;

(b) *Brevibacterium*; and

(c) *E. coli*.

16. The method of Claim 15, wherein microorganism B is auxotrophic or bradytrophic for valine and isoleucine.

17. The method of Claim 15, wherein step (b) mutagenesis is random chemical mutagenesis.

18. The method of Claim 15, wherein step (b) mutagenesis is site-specific mutagenesis of the ilvBN operon.

19. A process for producing an amino acid X comprising:

(a) culturing a microorganism C in a medium, wherein said microorganism C is obtained by the following method:

(i) selecting a parental microorganism A that produces a percent yield Y from dextrose of said amino acid;

(ii) mutagenizing said parental microorganism A to produce microorganism B by a method selected from the group consisting of:

(1) random chemical mutagenesis; and

(2) rDNA mutagenesis of the ilvBN operon;

(iii) selecting from step (b) at least one mutagenized microorganism B that is auxotrophic for one or more of the branched chain amino acids leucine, isoleucine and valine; and

(iv) selecting from step (c) at least one microorganism C which is an auxotroph that produces said amino acid X from dextrose in percent yield Z, wherein said percent yield Z is greater than said percent yield Y; and

(b) recovering said amino acid X that is produced from said microorganism C.

20. The process of claim 19, wherein amino acid X is selected from the group consisting of:

(a) glycine;
(b) alanine;
(c) methionine;
(d) phenylalanine;
(e) tryptophan;
(f) proline;
(g) serine;
(h) threonine;
(i) cysteine;
(j) tyrosine;
(k) asparagine;
(l) glutamine;
(m) aspartic acid;
(n) glutamic acid;
(o) lysine;
(p) arginine; and
(q) histidine.

21. The process of claim 20, wherein microorganism A is selected from the group consisting of:

(a) *Corynebacterium*;

(b) *Brevibacterium*; and

(c) *E. coli*.

22. The process of Claim 21, wherein microorganism B is auxotrophic or bradytrophic for valine and isoleucine.

23. The process of Claim 21, wherein step (ii) mutagenesis is random chemical mutagenesis.

24. The process of Claim 21, wherein step (ii) mutagenesis is site-specific mutagenesis of the ilvBN operon.

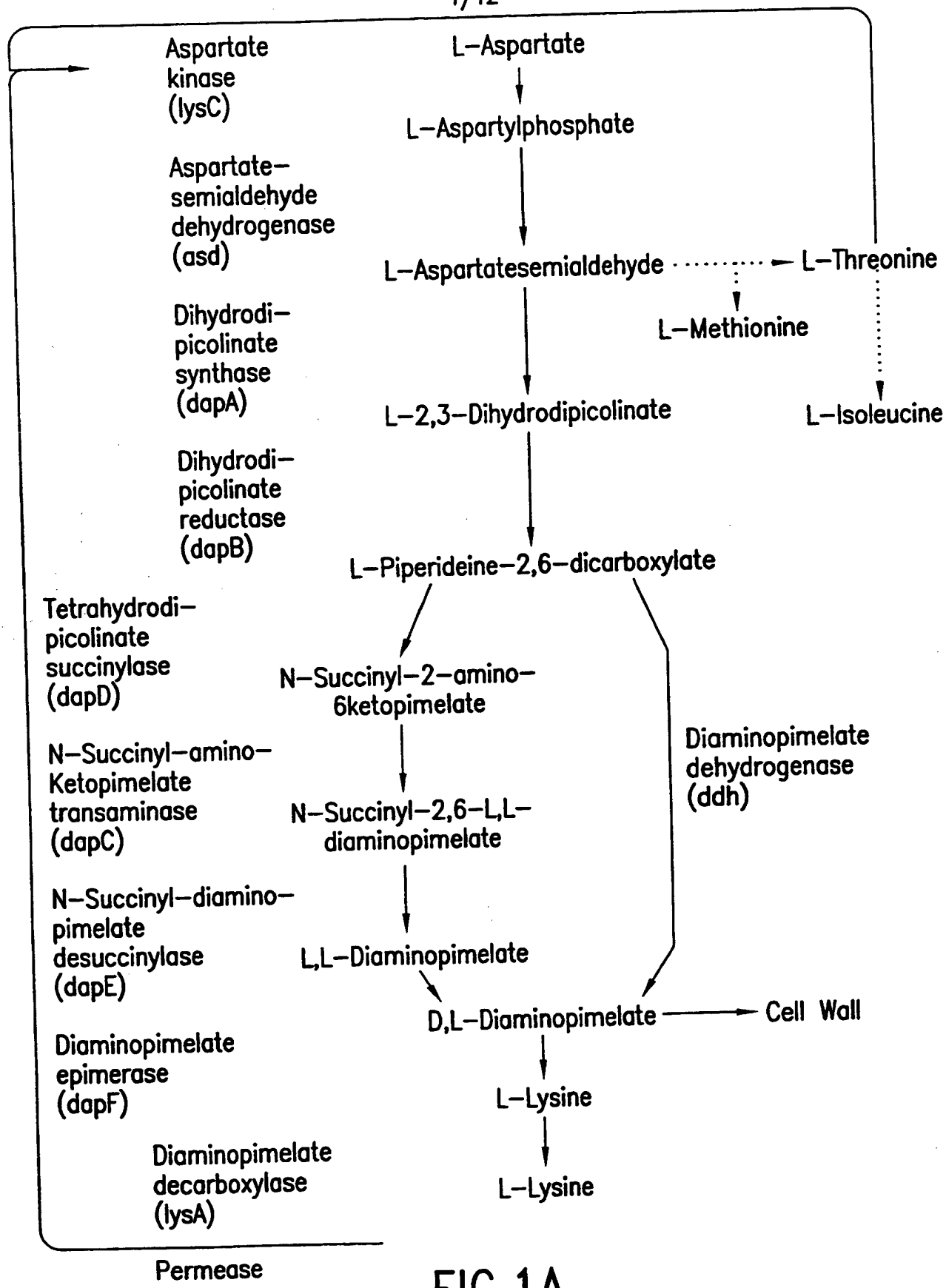
Aspartate kinase (lysC)
L-Aspartate
L-Aspartylphosphate
Aspartate-semialdehyde dehydrogenase (asd)
L-Aspartatesemialdehyde
L-Threonine
L-Methionine
Dihydrodi-picolinate synthase (dapA)
L-2,3-Dihydrodipicolinate
L-Isoleucine
Dihydrodi-picolinate reductase (dapB)
L-Piperideine-2,6-dicarboxylate
Tetrahydrodi-picolinate succinylase (dapD)
N-Succinyl-2-amino-6ketopimelate
Diaminopimelate dehydrogenase (ddh)
N-Succinyl-amino-Ketopimelate transaminase (dapC)
N-Succinyl-2,6-L,L-diaminopimelate
N-Succinyl-diamino-pimelate desuccinylase (dapE)
L,L-Diaminopimelate
D,L-Diaminopimelate
Cell Wall
Diaminopimelate epimerase (dapF)
L-Lysine
Diaminopimelate decarboxylase (lysA)
L-Lysine
Permease

FIG.1A

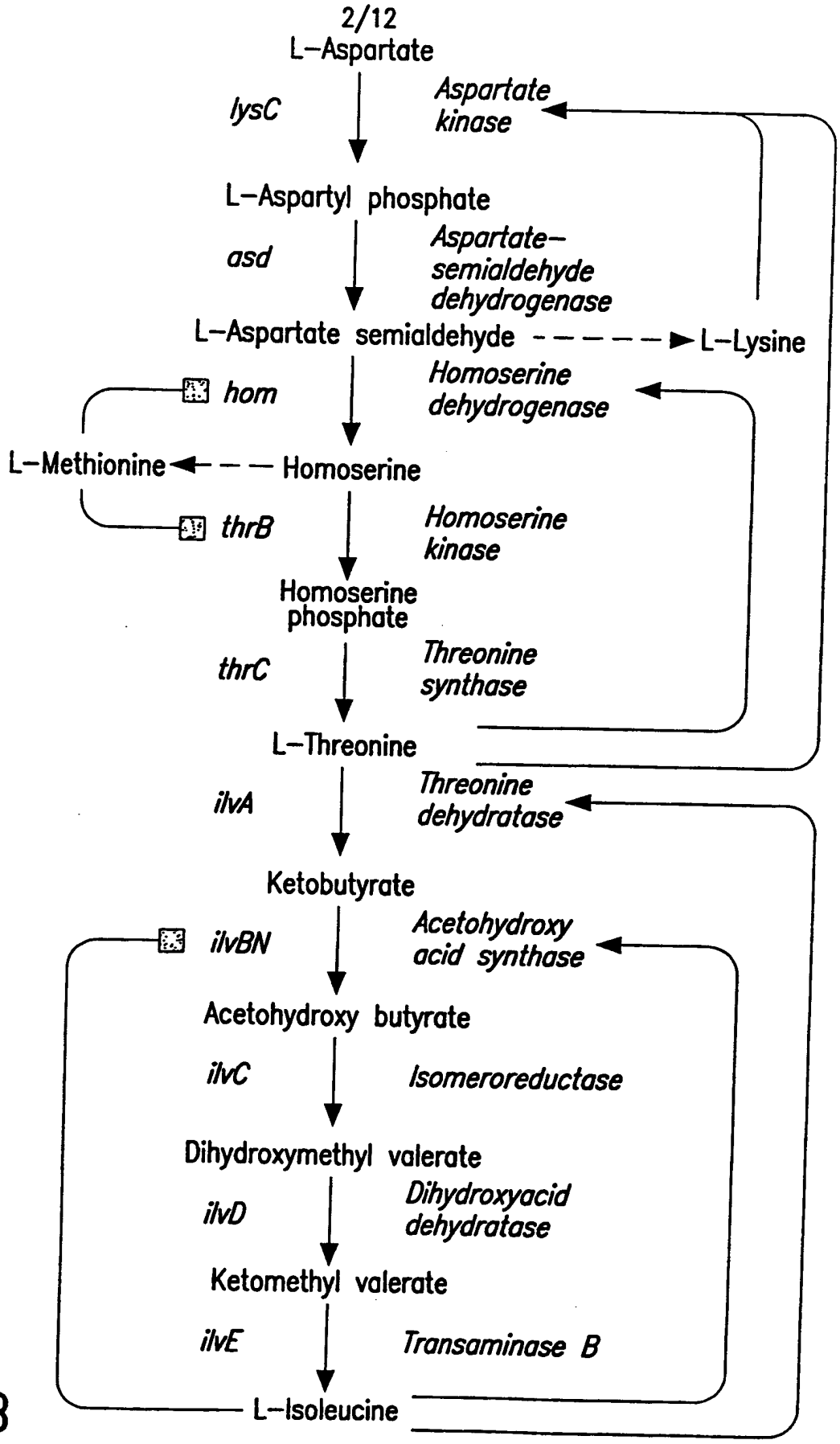

FIG.1B

WILD-TYPE ILVB NUCLEOTIDE SEQUENCE

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cctgagcggatgacaggtgcaaaggcaattgttcgatcgc 120
tcgaggagcttaacgccgacatcgtgttcggtattcctgg 160
tggtgcggtgctaccggtgtatgacccgctctattcctcc 200

       210       220       230       240
acaaaggtgcgccacgtcttggtgcgccacgagcagggcg 240
caggccacgcagcaaccggctacgcgcaggttactggacg 280
cgttggcgtctgcattgcaacctctggcccaggagcaacc 320
aacttggttaccccaatcgctgatgcaaacttggactccg 360
ttcccatggttgccatcaccggccaggtcggaagtggcct 400

       410       420       430       440
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atcaccatgccagtgaccaagcacaacttcatggtcacca 480
accctaacgacattccacaggcattggctgaggcattcca 520
cctcgcgattactggtcgccctggccctgttctggtggat 560
attcctaaggatgtccagaacgctgaattggatttcgtct 600

       610       620       630       640
ggccaccaaagatcgacctgccaggctaccgcccagtttc 640
aacaccacatgctcgccagatcgagcaggcagtcaagctg 680
atcggtgaggccaagaagcccgtcctttacgttggtggtg 720
gcgtaatcaaggctgacgcacacgaagagcttcgtgcgtt 760
cgctgagtacaccggcatcccagttgtcaccaccttgatg 800
```

FIG.2A

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WILD-TYPE ILVB NUCLEOTIDE SEQUENCE

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actgcagcgcagcgacctgctgattgctatcggctcccgc  920
tttgatgaccgcgtcaccggtgacgttgacaccttcgcgc  960
ctgacgccaagatcattcacgccgacattgatcctgccga 1000

        1010       1020       1030       1040
aatcggcaagatcaagcaggttgaggttccaatcgtgggc 1040
gatgcccgcgaagttcttgctcgtctgctggaaaccacca 1080
aggcaagcaaggcagagaccgaggacatctccgagtgggt 1120
tgactacctcaagggcctcaaggcacgtttcccgcgtggc 1160
tacgacgagcagccaggcgatctgctggcaccacagtttg 1200

        1210       1220       1230       1240
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cagttcgttgactttgaaaagccacgcacctggctcaact 1320
ccggtggactgggcaccatgggctacgcagttcctgcggc 1360
ccttggagcaaaggctggcgcacctgacaaggaagtctgg 1400

        1410       1420       1430       1440
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        1610       1620       1630       1640
cctttctgagggacttggctgtgttgccatccgcgtcacc 1640
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agatcaacgaccgcccagtagtcatcgacttcatcgtcgg 1720
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tccaactccgatatccagtacgcactcggattgcgcccat 1800

        1810       1820       1830       1840
tctttgatggtgatgaatctgcagcagaagatcctgccga 1840
cattcacgaagccgtcagcgacattgatgccgccgttgaa 1880
tcgaccgaggcatag 1895
```

FIG.2B

WILD-TYPE ILVB AMINO ACID SEQUENCE

```
        10        20        30        40
MNVAASQQPTPATVASRGRSAAPERMTGAKAIVRSLEELN  40
ADIVFGIPGGAVLPVYDPLYSSTKVRHVLVRHEQGAGHAA  80
TGYAQVTGRVGVCIATSGPGATNLVTPIADANLDSVPMVA 120
ITGQVGSGLLGTDAFQEADIRGITMPVTKHNFMVTNPNDI 160
PQALAEAFHLAITGRPGPVLVDIPKDVQNAELDFVWPPKI 200

       210       220       230       240
DLPGYRPVSTPHARQIEQAVKLIGEAKKPVLYVGGGVIKA 240
DAHEELRAFAEYTGIPVVTTLMALGTFPESHELHMGMPGM 280
HGTVSAVGALQRSDLLIAIGSRFDDRVTGDVDTFAPDAKI 320
IHADIDPAEIGKIKQVEVPIVGDAREVLARLLETTKASKA 360
ETEDISEWVDYLKGLKARFPRGYDEQPGDLLAPQFVIETL 400

       410       420       430       440
SKEVGPDAIYCAGVGQHQMWAAQFVDFEKPRTWLNSGGLG 440
TMGYAVPAALGAKAGAPDKEVWAIDGDGCFQMTNQELTTA 480
AVEGFPIKIALINNGNLGMVRQWQTLFYEGRYSNTKLRNQ 520
GEYMPDFVTLSEGLGCVAIRVTKAEEVLPAIQKAREINDR 560
PVVIDFIVGEDAQVWPMVSAGSSNSDIQYALGLRPFFDGD 600

       610       620       630       640
ESAAEDPADIHEAVSDIDAAVESTEA. 627
```

FIG.2C

AL 203Δ NUCLEOTIDE SEQUENCE

```
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ggagccagaaagtcgtgaatgtggcagcttctcaacagcc  40
cactcccgccacggttgcaagccgtggtcgatccgccgcc  80
cctgagcggatgacaggtgcaaaggcaattgttcgatcgc 120
tcgaggagcttaacgccgacatcgtgttcggtattcctgg 160
tggtgcggtgctaccggtgtatgacccgctctattcctcc 200

         210       220       230       240
acaaaggtgcgccacgtcttggtgcgccacgagcagggcg 240
caggccacgcagcaaccggctacgcgcaggttactggacg 280
cgttggcgtctgcattgcaacctctggcccaggagcaacc 320
aacttggttaccccaatcgctgatgcaaacttggactccg 360
ttcccatggttgccatcaccggccaggtcggaagtggcct 400

         410       420       430       440
gctgggtaccgacgctttccaggaagccgatatccgcggc 440
atcaccatgccagtgaccaagcacaacttcatggtcacca 480
accctaacgacattccacaggcattggctgaggcattcca 520
cctcgcgattactggtcgccctggccctgttctggtggat 560
attcctaaggatgtccagaacgctgaattggatttcgtct 600

         610       620       630       640
ggccaccaaagatcgacctgccaggctaccgcccagtttc 640
aacaccacatgctcgccagatcgagcaggcagtcaagctg 680
atcggtgaggccaagaagcccgtcctttacgttggtggtg 720
gcgtaatcaaggctgacgcacacgaagagcttcgtgcgtt 760
cgctgagtacaccggcatcccagttgtcaccaccttgatg 800
```

FIG.3A

AL 203Δ NUCLEOTIDE SEQUENCE

```
        810        820        830        840
gctttgggtactttcccagagtctcacgagctgcacatgg  840
gtatgccaggcatgcatggcactgtgtccgctgttggtgc  880
actgcagcgcagcgacctgctgattgctatcggctcccgc  920
tttgatgaccgcgtcaccggtgacgttgcacaccttcgcgc  960
ctgacgccaagatcattcacgccgacattgatcctgccga 1000

        1010       1020       1030       1040
aatcggcaagatcaagcaggttgaggttccaatcgtgggc 1040
gatgcccgcgaagttcttgctcgtctgctggaaaccacca 1080
aggcaagcaaggcagagaccgaggacatctccgagtgggt 1120
tgactacctcaagggcctcaaggcacgtttcccgcgtggc 1160
tacgacgagcagccaggcgatctgctggcaccacagtttg 1200

        1210       1220       1230       1240
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ccgcgtcaccaaagcggaggaagtactgccagccatccaa 1280
aaggctcgagagatcaacgaccgcccagtagtcatcgact 1320
tcatcgtcggtgaagacgcacaggtatggccaatggtgtc 1360
tgctggatcatccaactccgatatccagtacgcactcgga 1400

        1410       1420       1430       1440
ttgcgcccattctttgatggtgatgaatctgcagcagaag 1440
atcctgccgacattcacgaagccgtcagcgacattgatgc 1480
cgccgttgaatcgaccgaggcataa 1505
```

FIG.3B

AL 203Δ AMINO ACID SEQUENCE

```
          10        20        30        40
MNVAASQQPTPATVASRGRSAAPERMTGAKAIVRSLEELN 40
ADIVFGIPGGAVLPVYDPLYSSTKVRHVLVRHEQGAGHAA 80
TGYAQVTGRVGVCIATSGPGATNLVTPIADANLDSVPMVA 120
ITGQVGSGLLGTDAFQEADIRGITMPVTKHNFMVTNPNDI 160
PQALAEAFHLAITGRPGPVLVDIPKDVQNAELDFVWPPKI 200

          210       220       230       240
DLPGYRPVSTPHARQIEQAVKLIGEAKKPVLYVGGGVIKA 240
DAHEELRAFAEYTGIPVVTTLMALGTFPESHELHMGMPGM 280
HGTVSAVGALQRSDLLIAIGSRFDDRVTGDVDTFAPDAKI 320
IHADIDPAEIGKIKQVEVPIVGDAREVLARLLETTKASKA 360
ETEDISEWVDYLKGLKARFPRGYDEQPGDLLAPQFVIETL 400

          410       420       430       440
SEGLGCVAIRVTKAEEVLPAIQKAREINDRPVVIDFIVGE 440
DAQVWPMVSAGSSNSDIQYALGLRPFFDGDESAAEDPADI 480
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```

FIG.3C

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RV1B5 NUCLEOTIDE SEQUENCE

```
        10         20         30         40
AGGAGCCAGAAAGTCGTGAATGTGGCAGCTTCTCAACAGC  40
CCACTCCCGCCACGGTTGCAAGCCGTGGTCGATCCGCCGC  80
CCCTGAGCGGATGACAGGTGCACAGGCAATTGTTCGATCG 120
CTCGAGGAGCTTAACGCCGACATCGTGTTCGGTATTCCTG 160
GTGGTGCGGTGCTACCGGTGTATGACCCGCTCTATTCCTC 200

       210        220        230        240
CACAAAGGTGCGCCACGTCCTAGTGCGCCACGAGCAGGGC 240
GCAGGCCACGCAGCAACCGGCTACGCGCAGGTTACTGGAC 280
GCGTTGGCGTCTGCATTGCAACCTCTGGCCCAGGCGCAAC 320
CAACTTGGTTACCCCAATCGCTGATGCAAACTTGGACTCC 360
GTTCCCATGGTTGCCATCACCGGCCAGGTCGGAAGTAGCC 400

       410        420        430        440
TGCTGGGTACCGATGCTTTCCAGGAAGCCGATATCCGCGG 440
CATCACCATGCCAGTGACCAAGCACAACTTCATGGTCACC 480
AACCCCAACGACATTCCACAGGCATTGGCTGAGGCATTCC 520
ACCTCGCGATTACTGGTCGCCCTGGTCCTGTTCTAGTGGA 560
TATCCCCAAGGATGTTCAGAACGCTGAATTGGATTTCGTC 600

       610        620        630        640
TGGCCACCAAAGATCGACCTGCCAGGCTACCGCCCAGTTT 640
CAACACCGCATGCTCGACAGATTGAGCAGGCTGTCAAACT 680
GATCGGTGAGTCTAAGAAGCCTGTCCTTTACGTTGGCGGC 720
GGCGTTATCAAGGCTGATGCCCACGAAGAGCTTCGTGCGT 760
TCGCTGAGCACACCGGCATTCCAGTTGTCACCACATTGAT 800
```

FIG.4A

RV1B5 NUCLEOTIDE SEQUENCE

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        810       820       830       840
GGCGCTGGGAACCTTCCCAGAGTCCCACGAGCTGCACATG  840
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CACTGCAGCGCAGCGACCTGCTGATTGCTATCGGCTCCCG  920
CTTTGATGACCGCGTCACCGGTGACGTTGACACTTTCGCA  960
CCTGATGCCAAGATCATTCACGCCGACATTGATCCTGCCG 1000

       1010      1020      1030      1040
AAATCGGCAAGATCAAGCAGGTTGAGGTTCCAATCGTGGG 1040
CGATGCCCGCGAGGTTCTTGCTCGTCTGCTCGAAACCACC 1080
AAGGCAAGCAAGGCAGAGTCTGAGGACATCTCCGAGTGGG 1120
TTGACTACCTCAAGGGCCTCAAGGCACGTTTCCCACGTGG 1160
CTACGACGAGCAGCCAGGCGATCTGCTGGCACCACAGTTT 1200

       1210      1220      1230      1240
GTCATTGAAACCCTGTCCAAGGAAGTTGGCCCCGACGCAA 1240
TTTACTGCGCCGGCGTTGGCCAGCACCAGATGTGGGCAGC 1280
TCAGTTCGTTGACTTCGAAAAGCCACGCACCTGGCTCAAC 1320
TCCGGTGGACTGGGCACCATGGGCTACGCAGTTCCTGCGG 1360
CTCTTGGAGCAAAGGCTGGCGCACCTGACAAGGAAGTCTG 1400

       1410      1420      1430      1440
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GAACTCACCACCGCCGCAGTTGAAGGTTTCTCCATTAAGA 1480
TCGCACTAATCAACAACGGAAACCTGGGTATGGTTCGCCA 1520
ATGGCAGACCCTATTCTATGAAGGACGGTACTCAAATACT 1560
AAACTTCGTAACCAGGGCGAGTACATGCCCGACTTTGTTA 1600

       1610      1620      1630      1640
CCCTTTCTGAGGGACTTGGCTGTGTTGCCATCCGCGTCAC 1640
CAAAGCGGAGGAAGTACTGCCAGCCATCCAAAAGGCTCGA 1680
GAGATCAACGACCGCCCAGTAGTCATCGACTTCATCGTCG 1720
GTGAAGACGCACAGGTATGGCCAATGGTGTCTGCTGGATC 1760
ATCCAACTCCGATATCCAGTACGCACTCGGATTGCGCCCA 1800
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FIG.4B

RV1B5 NUCLEOTIDE SEQUENCE

1810 1820 1830 1840

TTCTTTGATGGTGATGAATCTGCAGCAGAAGATCTGCCGA 1840
CATTCACGAAGCCGTCAGCGACATTGATGCCGCCGTTGAA 1880
TCGACCGAGGCATAAGGAGAGACCCAAGATGGCTAATTCT 1920
GACGTCACCCGCCACATCCTGTCCGTACTCGTTCAGGACG 1960
TAGACGGAATCATTTCCCGCGTATCAGGTATGTTCACCCG 2000

2010 2020 2030 2040

ACGCGCATTCAACCTCGTGTCCCTCGTGTCTGCAAAGACC 2040
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GAGACCACTATCGCCCGCGCAATCATGCTGGTTAAGGTTT 2200

2210 2220 2230 2240

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ACTGATCCAATCCGGACAGATTGCACTCAACCGCGGTCCG 2400

2410 2420 2430 2440

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FIG.4C

RV1B5 AMINO ACID SEQUENCE

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ITGQVGSSLLGTDAFQEADIRGITMPVTKHNFMVTNPNDI 160
PQALAEAFHLAITGRPGPVLVDIPKDVQNAELDFVWPPKI 200

        210       220       230       240
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        410       420       430       440
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PVVIDFIVGEDAQVWPMVSAGSSNSDIQYALGLRPFFDGD 600

        610       620       630       640
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NRITVVVDADELNIEQITKQLNKLIPVLKVVRLDEETTIA 720
RAIMLVKVSADSTNRPQIVDAANIFRARVVDVAPDSVVIE 760
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FIG.4D

SEQUENCE LISTING

<110> Archer-Daniels-Midland Company
Rayapati, P. John
Crafton, Corey M.

<120> Metabolic Engineering of Amino Acid Production

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<150> US 60/146,379
<151> 1999-08-02

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            20                  25                  30

Val Arg Ser Leu Glu Glu Leu Asn Ala Asp Ile Val Phe Gly Ile Pro
        35                  40                  45

Gly Gly Ala Val Leu Pro Val Tyr Asp Pro Leu Tyr Ser Ser Thr Lys
    50                  55                  60

Val Arg His Val Leu Val Arg His Glu Gln Gly Ala Gly His Ala Ala
65                  70                  75                  80

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 00/20979

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 C12N1/21 C12P13/04 C12R1/13 C12R1/15
C12R1/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3 700 557 A (NAKAYAMA KIYOSHI ET AL) 24 October 1972 (1972-10-24) cited in the application column 1, line 68 -column 2, line 19	1-3,5,7, 13-15, 17, 19-21,23
X	EP 0 780 476 A (DEGUSSA) 25 June 1997 (1997-06-25) page 1, line 49 - line 53	1-3,5,7, 13-15, 17, 19-21,23
X	US 3 527 672 A (KUBOTA KOJI ET AL) 8 September 1970 (1970-09-08) column 1, line 32 - line 40	1-5, 13-17, 19-23

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 2000	25/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bilang, J

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 00/20979

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 205 849 A (TORAY INDUSTRIES) 30 December 1986 (1986-12-30) page 2, paragraph 4 -page 9, paragraph 2; table 8	1,2,13, 14,19,20
X	JP 63 173592 A (MITSUI TOATSU CHEM INC) 18 July 1988 (1988-07-18) abstract	1,2,13, 14,19,20
X	EIKMANNS B J ET AL: "MOLECULAR ASPECTS OF LYSINE, THREONINE, AND ISOLEUCINE BIOSYNTHESIS IN CORYNEBACTERIUM GLUTAMICUM" ANTONIE VAN LEEUWENHOEK,DORDRECHT,NL, vol. 64, no. 2, 1993, pages 145-163, XP000918559	1-3,5,7, 13-15, 17, 19-21,23
Y	page 153, left-hand column, paragraph 2	6,8,9, 18,24
X	BROEER S ET AL: "STRAINS OF CORYNEBACTERIUM GLUTAMICUM WITH DIFFERENT LYSINE PRODUCTIVITIES MAY HAVE DIFFERENT LYSINE EXCRETION SYSTEMS" APPLIED AND ENVIRONMENTAL MICROBIOLOGY,US,WASHINGTON,DC, vol. 59, no. 1, 1993, pages 316-321, XP000675845 ISSN: 0099-2240 page 316, right-hand column, paragraph 3	1-3,5,7, 13-15, 17, 19-21,23
Y	KEILHAUER C ET AL: "ISOLEUCINE SYNTHESIS IN CORYNEBACTERIUM GLUTAMICUM: MOLECULAR ANALYSIS OF THE ILVB-ILVN-ILVC OPERON" JOURNAL OF BACTERIOLOGY,US,WASHINGTON, DC, vol. 175, no. 17, 1 September 1993 (1993-09-01), pages 5595-5603, XP000611312 ISSN: 0021-9193 page 5597, left-hand column, paragraph 4	6,8,9, 18,24

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INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/US 00/20979

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		DK 112441 B	16-12-1968
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EP 0205849 A	30-12-1986	JP 1945776 C	23-06-1995
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		DE 3682709 A	16-01-1992
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